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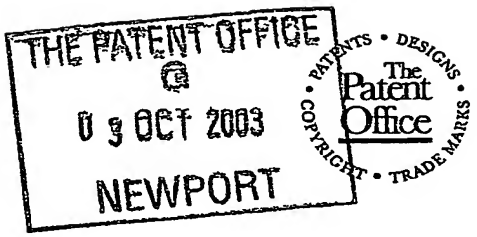

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1. Your reference — MJL/C1415.00/M

2. Patent application number *(The Patent Office will fill this part in)* — 0323147.9

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — Medical Research Council
20 Park Crescent
London W1B 1AL

   Patents ADP number *(if you know it)*

   If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom 596007001

4. Title of the invention — Improvements in or Relating to Concentration of Intracellular Agents

5. Name of your agent *(if you have one)* — Keith W Nash & Co

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — 90-92 Regent Street
Cambridge CB2 1DP

   Patents ADP number *(if you know it)* — 1206001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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Patents Form 1/77

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Continuation sheets of this form	
Description	19
Claim(s)	2
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11. I/We request the grant of a patent on the basis of this application.

Signature(s) Keith W. Nash & Co. Date 2/10/2003

12. Name, daytime telephone number and e-mail address, if any, of person to contact in the United Kingdom M J Lipscombe (01223) 355477

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C1415.00/M

Title: Improvements in or Relating to Concentration of Intracellular Agents

Field of the Invention

This invention relates to a method of inhibiting efflux proteins, pharmaceutical compositions for performing the method, and the use of certain active agents in the preparation of medicaments to inhibit efflux proteins.

Background of the Invention

Plasma membrane lipids are distributed asymmetrically in normal cells, with anionic phospholipids such as phosphatidylserine (PS) usually being confined to the inner leaflet of the plasma membrane. Energy is expended to maintain this asymmetry against a background of passive spontaneous, albeit slow, phospholipid diffusion between leaflets, and though no protein has uniquely been identified with the function of the inward transport of PS (aminophospholipid translocase activity), candidate proteins have been identified (Williamson and Schlegel, Biochim. Biophys. Acta 2002, 53-63).

Loss of membrane asymmetry with the consequent exposure of PS at the cell surface is known to occur (i) on anucleate 'cells' such as platelets and to a lesser extent erythrocytes as an integral part of the clotting cascade; and (ii) prior to membrane breakdown, being a marker of apoptotic cell death (Martin *et al*, 1995 J. Exp. Med. 182, 1545-1556; Vermes *et al*, 1995 J. Immunol. Methods 184, 39-51). Indeed binding of fluorescent annexin V to PS is the basis of commonly used commercially available assays for the detection of apoptotic cells. Reported exceptions to rule that PS exposure indicates apoptosis are rare, though has been found to occur during bicarbonate-stimulated 'capacitation' of sperm required for fertilisation (Gadella & Harrison, 2002 Biol. Reprod. 67, 340-350), whilst PS redistribution on macrophages is also believed necessary for engulfment of apoptotic cells (Marguet *et al*, 1999 Nat. Cell Biol. 1, 454-456). Other than in such exceptional cases,

PS exposure is generally assumed to mark a point in apoptosis beyond which cells cannot recover, and therefore to be irreversible.

Ablation of normal lipid asymmetry may happen by several means, none of which are well understood, and mechanisms may vary dependent on the stimulus. Rapid loss of lipid asymmetry, also known as lipid 'scrambling', can be stimulated experimentally by treatment with calcium ionophore. Whilst it was originally thought that the process was dependent on the activity of a phospholipid scramblase mediating non-specific bi-directional transport of phospholipids, knock-out mice, in which the gene encoding phospholipid Scramblase 1 (PLSCR 1) was deleted, exhibited no defect in PS externalisation (Zhou *et al*, 2002). More recently it has been suggested that the ATP binding cassette transporter, ABCA1 might act as an outwardly-directly PS translocase (Hamon *et al*, 2000), though whether this protein directly transports PS or acts upstream in the process is unclear.

Cell surface receptors for ATP can be divided into two classes: metabotropic and ionotropic. The ionotropic class (named "P2X" receptors), comprise seven known members, $P2X_1$ – $P2X_7$. They are ligand-gated ion channels. They are believed to be multisubunit proteins, with two transmembrane domains per subunit (Buell *et al*, 1996 Europ. J. Neurosci. 8, 2221).

$P2X_7$ is the most recently identified member of the P2X family. It is structurally related to the other P2X receptors, but has a significantly longer cytoplasmic C-terminus. Upon activation, the $P2X_7$ receptor opens a channel through the cell membrane, which channel is permeable to small cations (such as Na^+ or K^+). It is not yet clear whether the $P2X_7$ receptor itself actually forms the channel or acts on a heterologous pore-forming polypeptide.

Expression of the purinergic receptor $P2X_7$ is largely restricted to haematopoietic cells (Labasi *et al*, 2002 J. Immunol. 168, 6436-6445). Activation of $P2X_7$ receptors by ATP (or more potently benzoylbenzoyl ATP (BzATP)) induces reversible flopping of

phosphatidylserine (PS) to the outer leaflet of the cell membrane and release of IL-1β (MacKenzie *et al*, 2001 Immunity 5, 825-835). Genetic loss of $P2X_7$ receptors results in decreased IL-1β secretion and attenuation of inflammatory responses, whilst human polymorphisms in the $P2X_7$ gene are associated either with susceptibility to or survival of chronic lymphocytic leukemia (CLL). A further characteristic of $P2X_7$ receptor activation is that the lymphocyte plasma membrane becomes permeable to many fluorochromes of up to 300Da (Ralevic *et al*, 1998 Pharmacol. Rev. 50, 413-492). This has commonly been interpreted as indicating the conversion of a cation-selective channel into a cation-selective pore (e.g. as described in US 6,509,163).

The efflux of cationic hydrophobic molecules from mammalian cells is commonly mediated by a subset of ATP-binding cassette (ABC) transporters, including the multidrug resistance P-glycoprotein (P-gp; MDR1; ABCB1); members of the multidrug-resistance associated (MRP; ABCC) family of proteins; and the mitoxantrone resistance protein (ABCG2; also called breast cancer resistance protein, BCRP). The primary physiological role of these proteins is cellular defence against toxic molecules. However, these "efflux" proteins have an exceedingly broad range of substrates and also act to eliminate therapeutic drug compounds from target cells, thereby preventing the drug from attaining a therapeutically effective intracellular concentration. This phenomenon is especially observed, for example, in attempts to treat cancers with cytotoxic drugs, and represents a significant clinical obstacle.

Summary of the Invention

In its widest terms, the present invention provides a method of regulating the activity of one or more selected membrane proteins, the method comprising the step of contacting or mixing a lipid membrane comprising the selected membrane proteins with a substance which causes a rearrangement of at least part of the components of the membrane, which rearrangement results in an alteration of the activity of the selected proteins.

A "membrane protein" is a molecule which comprises one or more polypeptides and which is inserted within, or in some way attached to, a membrane lipid bilayer. The term

"membrane protein" is intended to encompass membrane lipoproteins, glycoproteins and other modified proteins. The lipid membrane is typically a eukaryotic cell membrane, but may also be part of a cell membrane system e.g. a membrane of the endoplasmic reticulum, golgi body, vesicle etc. or may be a synthetic (acellular) membrane, such as a liposome.

Eukaryotic cell membranes typically comprise a complex mix of lipids, glycolipids, phospholipids and the like. The present inventors have found that (a) it is possible to cause a rearrangement of at least part of the components of the membrane and that (b) such rearrangement can alter the activity of membrane proteins on or in the membrane.

The rearrangement of the components of the membrane may, in particular, involve a redistribution between the inner and outer leaflets of the membrane bilayer. More specifically, the rearrangement preferably involves at least one phospholipid, preferably phosphatidylserine (PS). In at least one embodiment, the invention involves contacting a cell membrane with a substance which causes a (preferably reversible) net redistribution of phosphatydylserine from the inner leaflet to the outer leaflet. This redistribution of phosphatidylserine can be prolonged by contacting the cell membrane with a further substance which binds to phosphatidylserine and retains it in the outer leaflet. An example of such a further substance is Annexin V or a PS-specific antibody or fragment thereof.

The membrane protein whose activity is regulated may be any membrane protein which is affected by the rearrangement of lipid (which term, in the present context, also encompasses phospholipid, glycolipid etc). One particular group of proteins which may be regulated in this way includes "efflux proteins" such as P-glycoprotein. The method of the invention may be used either to up- or down-regulate activity of the membrane proteins. The inventors propose that there are three mechanisms whereby loss of membrane asymmetry might affect any given plasma membrane-associated function:

i) changes in direct interaction between lipids and proteins;

ii) changes in indirect effect of plasma membrane on protein-protein interactions; and

iii) interaction of molecules with, and passive diffusion through, the plasma membrane.

As an illustration, it is known for example that membrane lipids play a central role in signal transduction through their interaction with proteins. There are many examples of this, e.g.

a) The membrane lipid phosphatidylinositol (4,5) P_2 is a substrate for phospholipase γ and β, its hydrolysis (following receptor activation) resulting in the production of the key signalling molecules inositol triphosphate and diacylglycerol.

b) The sphingolipid metabolites ceramide, sphingosine and sphingosine 1-phosphate are regulators of cell proliferation and apoptosis. Ceramide, for example is a potent apoptosis mediator, triggering several signalling pathways such as the stress-activated protein kinase cascade. As several antitumour agents induce ceramide production it follows that any alteration of drug/membrane interactions may modulate cytotoxic effects independent of the level of intracellular drug accumulation.

c) Most protein kinase C (PKC) isoforms require PS and calcium for their activation. It follows that a reduction of the concentration of PS in the cytoplasmic leaflet of the plasma membrane is likely to modulate PKC-dependent activities.

In one specific embodiment the invention may be used, for example, to inhibit the activity of a membrane efflux protein.

One particular embodiment of the invention is discussed in greater detail below.

The present inventors have surprisingly discovered that activation or stimulation of the $P2X_7$ receptor has the effect of increasing the intracellular concentration of exogenously administered substances, such as therapeutic drugs. In many instances, but not necessarily all, this effect has been shown by the inventors to be mediated by inhibiting the action of cell membrane efflux proteins.

Thus in one aspect the invention provides a method of increasing the effective intracellular concentration of a therapeutic molecule within a cell expressing a $P2X_7$ receptor, the method comprising the step of contacting the cell with the therapeutic molecule and with a substance which stimulates the $P2X_7$ receptor. In a preferred embodiment the invention provides a method of inhibiting the action of a cell membrane efflux protein, the method comprising the step of contacting a cell expressing a $P2X_7$ receptor with a substance which causes activation of the $P2X_7$ receptor.

A number of efflux proteins are known. These are generally ATP-binding cassette ("ABC") transporter proteins, and include: P-glycoprotein (also known as P-gp, MDR1 or multidrug resistance 1; and ABCB1); members of the multidrug-resistance associated family of proteins (exemplified by MRP and ABCC); and mitoxantrone resistance proteins (ABCG2, also known as breast cancer resistance protein, BRCP).

The method of the invention may be performed *in vitro* or *in vivo*. Use of the method *in vitro* may be advantageous, for example, to facilitate the study of the effect of a particular substance on a cell or tissue, which substance might otherwise be removed from the cell by efflux proteins so as to prevent an effective concentration from being attained. Use of the method *in vivo* may be especially advantageous to facilitate delivery of therapeutic drugs to intracellular targets.

Substances which stimulate the $P2X_7$ receptor include $P2X_7$ agonists such as ATP and ATP analogues, such as benzoylbenzoyl ATP (abbreviated as BzATP). These compounds are stimulatory for the $P2X_7$ receptor at concentrations which are non-toxic. BzATP is

preferred to ATP, as being both more specific for the $P2X_7$ receptor, and possessing greater receptor-stimulating activity.

Other substances which the inventors predict might stimulate the $P2X_7$ receptor include antibodies and antibody-like variants (such as scFv, Fab etc) with specific binding affinity for the $P2X_7$ receptor. More especially divalent or multivalent antibodies and the like are preferred. Binding of antibodies or antibody-like molecules to the surface-exposed portion of the $P2X_7$ receptor is predicted to cause stimulation thereof. The use of such $P2X_7$ stimulants may be advantageous, since they can specifically target $P2X_7$, whereas more conventional agonists such as ATP will have a wide range of effects in addition to stimulating the $P2X_7$ receptor.

Antibodies specific for the $P2X_7$ receptor can readily be made by the person skilled in the art. Recombinant $P2X_7$ can, for instance, be readily expressed in a suitable host cell (e.g. as described in US 6,509,163). The protein can be readily purified e.g. by epitope tagging and passage through an affinity chromatography column. A hexahistidine tag is commonly used for this purpose. The purified protein can then be used to generate polyclonal antisera or monoclonal antibodies by conventional techniques (e.g. as described by Sambrook *et al*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, USA).

As explained above, the inventors have surprisingly found that stimulation of the $P2X_7$ receptor results in inhibition of various efflux proteins, including in particular P-gp. Accordingly the method of the first aspect of the invention may be especially useful in facilitating chemotherapeutic treatment, by increasing the effective intracellular concentration of drugs within cells which express the $P2X_7$ receptor and an efflux protein which is inhibited by $P2X_7$ receptor stimulation.

Accordingly in a preferred embodiment, the invention provides a method of increasing the effective intracellular concentration of a therapeutic molecule within a cell expressing a $P2X_7$ receptor and an efflux protein which is inhibited by stimulation of the $P2X_7$ receptor,

the method comprising the steps of contacting the cell with the therapeutic molecule and with a substance which stimulates the $P2X_7$ receptor. It will be appreciated that, in this context, the therapeutic molecule may be a cytotoxic drug intended to kill the cell (e.g. because the cell is malignantly transformed), so the term "therapeutic" should be construed accordingly.

It will be appreciated that the $P2X_7$ receptor stimulating substance may be co-administered or administered separately. The time interval between administration of the two agents which is permissible will depend, at least in part, on the pharmacokinetic profile (e.g. half-life *in vivo*) of the therapeutic molecule.

The $P2X_7$ receptor is expressed primarily on haemopoietic cells, such as lymphocytes, macrophages, and the like. Thus, the invention will be especially useful in facilitating treatment of disease in these cells. Examples of particular interest are the facilitation of treatment of malignancies such as leukaemias, autoimmune disorders and allergies. Numerous drugs have been used to treat diseases of this sort, and which are known, or suspected, of being exported from the cell by efflux proteins such as P-gp.

Examples of such drugs include the following: cimetidine, colchicine, cyclosporin, dexamethasone, mitomycin, terfernadine, vinblastine, simvastatin and vincristine. This is a non-exhaustive list, and there are many other drugs which may benefit from the present invention by administration generally simultaneously with a $P2X_7$ receptor stimulating substance.

For present purposes, a drug and a $P2X_7$ receptor stimulating substance may be considered to be administered "generally simultaneously" if there is a time point in which both substances are present in the subject at an effective concentration.

The drug may be administered by any conventional route e.g. intravenous, intramuscular or subcutaneous injection; or by nasal, oral or rectal administration. The $P2X_7$ receptor

stimulating substance may likewise be administered by a conventional route as aforementioned, although generally an injectable route will be preferred.

A suitable dose of $P2X_7$ receptor stimulating substance will depend on the activity of the substance and the route of administration. For example, BzATP has been injected into the hindpaw of rats at a concentration of 100nmol/50µl and found to be non-toxic (Wismer *et al*, 2003 Brain Research 965, 187-193). A suitable dose in a human subject would probably be such as to achieve a plasma concentration of 10nmol-10mM, preferably in the range 100nmol-1mM. An amount suitable to achieve this concentration can readily be determined by routine trial and error experimentation.

Use of the method of the invention to inhibit efflux proteins enables a higher effective intracellular concentration of drug to be attained within $P2X_7$-expressing cells for a given dose of drug. Alternatively, the dose of drug can be reduced and the effective intracellular concentration can be maintained – this is likely to reduce the severity and/or incidence of adverse reactions to the drug.

In another aspect the invention provides a pharmaceutical composition for administration to a mammalian subject, the composition comprising: a therapeutic drug; a $P2X_7$ receptor-stimulating substance; and a physiologically acceptable carrier, diluent or excipient. Numerous suitable carriers, diluents or excipients are known to those skilled in the art, and include for example sterile water, saline, phosphate-buffered saline, calcium carbonate, starch, gelatin and the like.

In a further aspect the invention provides a method of making a pharmaceutical composition, comprising the step of combining in admixture a therapeutic drug, a $P2X_7$ receptor-stimulating substance, and a physiologically acceptable carrier, diluent or excipient.

In yet another aspect the invention provides for the use of a $P2X_7$ receptor-stimulating substance in the preparation of a pharmaceutical composition to inhibit an efflux protein in $P2X_7$ receptor expressing cells.

It is conceivable that rearrangement of lipid within the cell membrane of lymphocytes can be achieved by means which do not require stimulation of the $P2X_7$ receptor. For example, binding of a ligand to the T cell receptor (TCR) may trigger lipid rearrangement. The ligand may be for example an antibody or antibody-like molecule with specific binding activity for the TCR. Alternatively antigen-stimulation may possibly have a similar effect – this would be extremely useful for causing lipid rearrangement in the cell membrane of B cells, since B cells express the $P2X_7$ receptor at only a relatively low level, so $P2X_7$ receptor stimulation is not as effective in B cells as in T cells.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, in which:

Figure 1A shows a graph of annexin V binding (as judged by mean fluorescence) against time for $CD4^+$, $CD8^+$ and $CD4^-$ $CD8^-$ (primarily B) cells;

Figure 1B shows inhibition of P-gp activity (as measured by increased Rh123-mediated fluorescence) against time for the same three different cell populations;

Figure 1C is a bar chart comparing Rh123 uptake in cells with or without treatment with BzATP;

Figure 1D shows graphs of BODIPY-taxol uptake (as judged by fluorescence) against time for NIH-3T3 cells or NIH-3T3-MDR cells, which do not express significant levels of the $P2X_7$ receptor;

Figures 2(i) and 2(ii) a/b are graphs showing binding or uptake, as appropriate, (against time) of particular fluorochrome-labelled substances by various cell populations under different experimental conditions;

Figures 3(i)-(iii) column A are graphs showing BODIPY-taxol uptake against time for three different cell populations. Figures 3(i)-(iii) column B show labelled annexin V binding (a measure of PS translocation) against time for the same cell populations as in column A;

Figures 3C(i) and (ii) are an alternative representation of some of the data shown in Figures 3(ii)A and 3(iii)A;

Figure 3D is a graph of number of cells against P-gp expression;

Figures 4A-D are histograms showing uptake/binding of various fluorochromes by cell populations under different experimental conditions; and

Figures 5A-C are graphs showing uptake (against time in seconds) of particular fluorochromes by various cell populations under different experimental conditions.

Examples

1. **MATERIALS AND METHODS**

   Mice

   Mdr1a/mdr1b double knockout mutations in mice (Schinkel *et al*, 1997 Proc. Natl. Acad. Sci. USA 94, 4028-4033) were backcrossed for at least seven generations onto the FVB background at Taconic farms (Germantown, USA). Mice were between 6 and 14 weeks of age. When experiments utilised FVB.mdr1a/b$^{-/-}$ mice, age-matched FVB mice were used.

Cells

NIH 3T3 cells stably transfected with the human *MDR1* gene (Ueda *et al*, 1987 Proc. Natl. Acad. Sci. USA 84, 3004-3008) were a gift from Michael Gottesman (NIH, Bethesda) and were maintained in 1μg/ml colchicine in Dulbecco's modified Eagle's medium (DMEM – Sigma). HEK 293 cells stably transfected with $rP2X_7$ have been described elsewhere (Wilson *et al*, 2002 J. Biol. Chem. 277, 34017-34023). Transient transfection of HEK293 cells was performed using polyethyleneimine (Sigma), as described previously (Dixon *et al*, 2000 Hum. Mol. Genet. 9, 1209-1217). pMDR1-wt, which encodes wild-type P-gp, has also been described previously (Blott *et al*, 1999 EMBO J. 18, 6800-6808). Expression of the *MDR1* gene product, P-gp, on transfected cells was determined by labelling of cells with $UIC2^{PE}$ in the presence of cyclosporine A (Sigma), as described by Mechetner *et al*, (1997 Proc. Natl. Acad. Sci. 94, 12908-12913).

Fluorochrome efflux assays

Murine mesenteric lymph nodes (10^7/ml) were disaggregated in DMEM. To discriminate between different lymphocyte subsets, cells were stained with $CD4^{APC}$, $CD4^{CYCHROME}$, $CD4^{PE}$, $CD4^{FITC}$, $CD8^{APC}$, $CD8^{PE}$, $CD8^{PERCP}$, $CD8^{FITC}$, $B220^{APC}$ (Becton Dickinson, CA) antibodies as indicated. The fluorophore on antibodies used to identify $CD4^+$ and $CD8^+$T cells was varied in some experiments to avoid overlap between emission spectra of the antibody-conjugated fluorochromes and fluorescent transport substrates. Cells were washed with DMEM and analysed by flow cytometry (CellQuest software – Becton Dickinson). Live cells were gated by eye on the basis of forward scatter and side scatter. Lymphocytes lacking CD4 and CD8 were defined as B cells.

To measure drug uptake the following fluorochromes were used (from Molecular Probes, Leiden, Netherlands, unless stated): 0.2 μM BODIPY-taxol (BT), 0.25 μM Rh123, 0.2 μM mitoxantrone (Sigma). PS exposure was monitored simultaneously in the same population of cells by increased binding of annexin V as follows. Cells were equilibrated with annexin V^{FITC}, annexin V^{PE}, or annexin V^{CY5} (AV- Becton

Dickinson, CA) together with propidium iodide (PI) for four minutes, and analysed by flow cytometry on a FACScalibur machine using CellQuest software (Becton Dickinson, CA). Baseline fluorescence was established for approximately one minute prior to addition of 150 μM (unless otherwise stated) 2'-3'-O-(4-benzoylbenzoyl)-adenosine 5'-triphosphate (BzATP, Sigma). Cells were monitored for BT uptake or PS exposure continuously in real time for up to 15 minutes, as indicated. Continuous line graphs of changes in annexin V binding and fluorochrome uptake were plotted using FCSPress software (FCSPress.com, Cambridge, UK).

2. Murine T lymphocytes express both the $P2X_7$ receptor (Chused *et al*, 1996 J. Immunol. 157, 1371-1380) and P-gp (Bommhardt *et al*, 1994 Eur. J. Immunol. 24, 2974-2981). To assess whether $P2X_7$-stimulation results in PS translocation by murine T cells, and the potential effect of phosphatidylserine (PS) translocation on P-gp function, the inventors monitored both PS translocation and the uptake of the P-gp substrate Rh123, simultaneously in the same cell population, using real-time flow cytometry. The results of these experiments (illustrated in Figures 1A-D) showed that activation of the $P2X_7$ receptor simultaneously stimulates PS translocation within the cell membrane and inhibits P-gp actvity.

Lymphocytes were labelled with anti-$CD4^{CYCHROME}$ and anti-$CD8^{APC}$ antibodies to discriminate between $CD4^+$T cells, $CD8^+$T cells and B cells ($CD4^-$ $CD8^-$). The lymphocytes were incubated with annexin V^{PE}, and then 0.25μM Rh123 and 150μM BzATP were added sequentially (as indicated in the Figures by arrows).

Figure 1A shows increases in extracellular PS exposure in each cell population, as indicated by increased biding of annexin V^{PE}. It is clear from Figure 1A that BzATP stimulated PS translocation with the order of responsiveness $CD4^+$ cells > $CD8^+$ T cells >> B cells (Fig. 1A), consistent with the known pattern of expression of $P2X_7$ receptors.

Figure 1B shows P-gp activity, measured simultaneously in the same population of cells, as indicated by uptake of Rh123 (increase in mean (± S.E.) fluorescence). Note that an increase in Rh123 uptake (fluorescence increase) reflects an inhibition of P-gp activity. Again, the order of responsiveness was $CD4^+ > CD8^+ >> B$ cells.

Figure 1C shows the results obtained when lymphocytes were labelled with anti-$CD4^{PE}$, incubated with annexin V^{CY5} and then 0.2μM Rh123 added in the presence (n=5) or absence (n=4) of 150μM BzATP. (The histogram shows the uptake of Rh123 (increase in mean (±S.D.)) at 3 minutes.)

Figure 1D shows that BzATP does not affect P-gp activity in NIH3T3 cells which lack the $P2X_7$ receptor. BODIPY-taxol (BT) and 175μM BzATP were added to NIH3T3-MDR and parental NIH3T3 cells at times indicated by arrows. BT uptake was monitored by continuous FACS analysis, as an increase in fluorescence as a function of time. As expected, BT did not accumulate in NIH3T3-MDR cells due to P-gp activity. In contrast to lymphocytes (Figures 1A and B), BzATP did not inhibit P-gp activity and thus did not increase BT accumulation. (Note: in these and other experiments utilising cultured cells, a small proportion of cells rapidly accumulated very high levels of BODIPY-taxol and were unresponsive to BzATP; it was unclear what such cells (or cell debris) represented, and they were not analysed further).

3. In a further experiment, lymphocytes from mdrla/b-deficient or parental mice (mdrla/$b^{+/+}$) were labelled with anti-$CD8^{PE}$ and anti-$CD8^{PERCP}$ antibodies, respectively (to discriminate between $CD8^+$ cells from each mouse during subsequent analysis). The cells from each mouse were mixed, and BODIPY-taxol and annexin V^{CY5} added to enable simultaneous monitoring by flow cytometry of PS translocation and P-gp activity in real time in the same population of cells. Reduced BT uptake is indicative of enhanced P-gp activity; increased $annexin^{CY5}$ binding indicates increased PS translocation from the inner to outer leaflet of the cell membrane. The results are shown in Figures 2(i) and (ii).

Figures 2(i) and 2(ii) (a/b) are graphs showing BT uptake (as measured by fluorescence) against time for mdr1a/b-deficient mice (column A) or parental mice (column B). Figure 2(i) shows the BT uptake in untreated cells. It is apparent that BT uptake in the cells from the parental mouse is much lower than that in cells from the mdr-deficient mice, as expected, indicating that BT uptake is limited by P-gp as reported previously (Binaschi *et al*, 1995 Int. J. Cancer 62, 84-89).

Figures 2(ii) (a) and (b) show, respectively, BT uptake and PS translocation following stimulation by addition of BzATP. BT uptake following BzATP treatment rapidly reached equivalence in the mdr-deficient and wild type cells. (Note that, at the concentration of BzATP employed, a minority of cells failed to translocate PS: in these cells BT uptake remained greater in the mdr-deficient cells, thus strengthening the correlation between PS translocation and inhibition of P-gp activity.)

4. To confirm that BzATP was acting through $P2X_7$ receptors, the effect of $P2X_7$-stimulation on P-gp activity was studied on HEK 293 cells stably transfected with the $P2X_7$ gene. As HEK cells do not express P-gp, MDR1 cDNA was transiently transfected into the cells. The cells were equilibrated with annexin V^{CY} and propidium iodide. The results are shown in Figures 3(i)-(iii) which are graphs of fluorescence against time. Columns A and B show the results for BT uptake and PS translocation respectively. Only the responses of live cells (i.e. those excluding propidium iodide) were analysed.

BzATP induced PS translocation and concomitantly increased BODIPY-taxol uptake (i.e. inhibited P-gp) in cells expressing both P-gp and $P2X_7$ receptor (Fig. 3(ii)) but neither induced PS translocation nor increased BODIPY-taxol uptake in cells expressing P-gp but lacking $P2X_7$ receptors (Fig. 3(i)). Thus, only in $P2X_7$-expressing cells did BzATP inhibit P-gp activity, confirming that BzATP acts through the $P2X_7$ receptor.

As observed in Figure 1 a small proportion of cultured cells (or cell debris) rapidly accumulated very high levels of BT and were unresponsive to BzATP treatment. These were excluded from the histogram analysis presented in Figures 3C and D.

Figure 3C is an alternative plot of data in Figures 3(ii)A and 3(iii)A, and shows a comparison of steady-state BODIPY-taxol uptake by mock transfected $P2X_7$ cells ("X") and $P2X_7$-MDR cells ("Y"). (i) before BzATP stimulation (indicated by gates R3), or (ii) following BzATP stimulation (indicated by gates R4).

Figure 3D shows P-gp expression by mock-transfected HEK 293-$P2X_7$ cells ("X"), and HEK 293-$P2X_7$ cells transiently transfected with the *MDR1* gene ("Y") as measured by binding of fluorescently-conjugated, P-gp-specific antibody ($UIC2^{PE}$). An equivalent proportion (~35%) of $P2X_7$-MDR1 cells expressed P-gp (3D) as were able to exclude BODIPY-taxol (Fig. 3Ci).

5. In this example, the inventors compared the uptake of BODIPY-taxol by parental and mdr1a/b-deficient lymphocytes, briefly stimulated with BzATP either in the continuous presence of annexin V to 'trap' PS in the outer leaflet, or with annexin V added only shortly before acquisition. The results are shown in Figures 4A-D. The experiments were performed as described below.

Lymphocytes from mdrla/b-deficient or parental mice (mdrla/$b^{+/+}$) were labelled with anti-$CD8^{PE}$ and anti-$CD8^{CY}$ antibodies, respectively (to discriminate between $CD8^+$ cells from each mouse during subsequent analysis). The cells from each mouse were mixed, stimulated with BzATP for 45 seconds, washed, and incubated with annexin V either for 30 minutes to 'trap' PS in the outer leaflet (column A), or for only the final 4 of 30 minutes (column B) to allow detection of PS prior to acquisition. BODIPY-taxol uptake was then measured by real-time flow cytometry. Reduced BT uptake indicates enhanced P-gp activity; increased $annexin^{CY5}$ binding indicates increased PS translocation from the inner to outer leaflet of the membrane. Row C shows histograms comparing annexin V binding at steady state (after 180-220

seconds incubation) and Row D shows BODIPY-taxol accumulated by mdrla/b deficient (thick line) and mdrla/b$^{+/+}$ (thin line) lymphocytes in the same population of cells at the same timepoint gated on the basis of either high or low levels of external PS. As PS exposure in samples stained with annexin V for four minutes immediately following stimulation (not shown) was equivalent to that in cells incubated for 30 minutes, PS trapping by annexin V appeared to be effectively complete.

The data demonstrate that annexin V treatment prevents the restoration of P-gp activity.

6. P-glycoprotein is only one of a family of transporters that can provide protection from drugs (Allen *et al*, 2000 Cancer Res. 60, 5761-5766). The inventors therefore examined whether $P2X_7$-stimulation may also inhibit drug efflux activity by transporters other than P-gp. Mitoxantrone efflux, presumed to be mediated by the breast cancer resistance protein (abcg2), was found in $CD4^+$ (and to a lesser extent $CD8^+$ T cells). The results are shown in Figures 5A-C.

Figure 5A shows the results obtained when lymphocytes from mdrla/b-deficient mice were labelled with anti-$CD4^{FITC}$ to gate $CD4^+$ T cells. Mitoxantrone (Mx) or Mx plus BzATP, were added as indicted by arrows. Mitoxantrone uptake was measured as an increase in mean (± S.E.) fluorescence (FL-4 channel). Figure 5B shows results obtained when lymphocytes from mdrla/b-deficient mice were labelled with anti-$CD8^{PERCP}$, and BODIPY-taxol or BODIPY-taxol plus 175μM BzATP added as indicated. BODIPY-taxol uptake was measured as an increase in mean (± S.E.) fluorescence (FL-1 channel). Figure 5C similarly illustrates results obtained when HEK 293 and $P2X_7$-HEK 293 cells were equilibrated with propidium iodide. BODIPY-taxol (BT) and 150μM BzATP were added, as indicated. Only the responses of live cells (i.e. those excluding PI) were subsequently analysed.

The Figures show that BzATP stimulation of $P2X_7$ induced PS translocation and markedly increased uptake of mitoxantrone (MW 481) in mdrla/b-deficient lymphocytes, indicating that loss of plasma membrane asymmetry may be associated with inhibition of transporters in addition to P-gp. Though P-gp is the only characterised transporter for BODIPY-taxol, BzATP also increased the rate of uptake of this fluorochrome in mdrla/b-deficient $CD4^+$and $CD8^+$T cells, and in $P2X_7$.HEK 293, but not in control cells. Loss of membrane asymmetry may therefore also increase drug uptake by routes independent to effects on known multidrug transporters.

P-gp protects normal lymphocytes from toxins, and by pumping drugs from cells it can reduce the efficacy of chemotherapy. Expression of P-gp is most commonly associated with multidrug resistance on cancers, but it also provides potential barriers to the treatment of many other immune-related disorders from transplant rejection to chronic autoimmune disease and AIDS (Kim *et al*, 1998 J. Clin. Invest. 101, 289-294; Fellay *et al*, 2002 Lancet 359, 30-36). Concomitant with $P2X_7$-stimulated PS translocation, lymphocytes markedly increased uptake of the P-gp substrates Rh123(MW 381) and BODIPY-taxol (MW 1024), but not of the non-P-gp substrate propidium iodide (MW 414). Hence, $P2X_7$-stimulation results in selective increase in drug uptake consistent with loss of P-gp activity. The inventors' findings suggest the apparent increase in permeability is due (at least in part) to inhibition of efflux pumps. As $P2X_7$ activation increases 'flopping' of PS to the extracellular leaflet of the membrane simultaneously with the inhibition of P-gp activity, and as P-gp activity is known to be affected by the lipid composition of the membrane (Romsicki *et al*, 1999 Biochemistry 38, 6887-6896; Aswarakarn *et al*, 2001 J. Biol. Chem. 276, 38457-38463), a plausible interpretation of the data is that loss of plasma membrane lipid asymmetry inhibits P-gp activity by preventing the structural changes of the P-gp molecule required for drug efflux (Rosenberg *et al*, 2001 EMBO J. 20, 5615-5625). Alternatively, altered membrane asymmetry may alter the distribution of drugs in the lipid phase such that they cannot access the substrate biding site of P-gp (Higgins & Gottesman 1992 Trends Biochem. Sci. 17, 18-21).

It is reasonable to assume that $P2X_7$-stimulation results not only in the flopping of PS to the outer leaflet of the plasma membrane, but also a broader lipid redistribution. Were this not the case it is likely that the resulting excess phospholipid content in the outer leaflet would result in rapid membrane disruption. It is also likely that unidirectional lipid translocation would be highly energetically unfavourable. It follows that there is a high probability any process dependent on membrane lipids will be modulated by loss of lipid asymmetry.

The potential of pharmacological inhibitors of P-gp activity to reverse multidrug resistance in cancers has been severely limited by the consequent elevated drug entry into organs such as the brain that are protected by drug transporter activity (Schinkel 2001 Adv. Exp. Med. Biol. 500, 365-372). As $P2X_7$-stimulation inhibits the activity of P-gp and probably also of other drug transporters, and the receptor is relatively specific to lymphocytes, its agonists may increase therapeutic drug uptake in lymphocytes but not in other tissues. Co-administration of $P2X_7$ agonists would be expected to potentiate lymphoid-specific accumulation of a wide variety of drugs including anti-cancer agents, HIV protease inhibitors, and immunosuppressants. This should avoid the undesirable consequences of traditional multidrug inhibitors that lead to widely altered, and potentially cytotoxic, drug uptake.

BzATP-mediated stimulation did not result in an increased uptake of propidium ions (MW 414), (data omitted for brevity), hence the observed effects were not simply due to a non-specific increase in plasma membrane permeability.

Claims

1. A method of increasing the effective intracellular concentration of a therapeutic molecule within a cell expressing a $P2X_7$ receptor, the method comprising the step of contacting the cell with the therapeutic molecule and with a substance which stimulates the $P2X_7$ receptor.

2. A method according to claim 1, wherein the therapeutic molecule is a cytotoxic drug and it is desired to kill the cell.

3. A method according to claim 1 or 2, wherein the $P2X_7$ receptor stimulating substance comprises ATP, an analogue of ATP, or an immunoglobulin or immunoglobulin-like variant which possesses specific binding activity for the $P2X_7$ receptor.

4. A method according to any one of the preceding claims, wherein the cell expresses an efflux protein which is inhibited by stimulation of the $P2X_7$ receptor.

5. A method according to claim 4, wherein the efflux protein which is inhibited is one or more selected from the group consisting of: P-glycoprotein; mitoxantrone resistance protein; and a member of the multidrug-resistance associated family of proteins.

6. A method according to any one of the preceding claims, wherein the therapeutic molecule and the $P2X_7$ receptor stimulating substance are co-administered.

7. Use of a $P2X_7$ receptor stimulating substance in the preparation of a medicament to cause rearrangement of at least part of the lipid or phospholipid or glycolipid component of a cell membrane.

8. Use of a $P2X_7$ receptor stimulating substance in the preparation of a medicament to regulate the activity of a cell membrane protein.

9. Use of a $P2X_7$ receptor stimulating substance in the preparation of a medicament to inhibit an efflux protein in a cell.

10. Use of a $P2X_7$ receptor stimulating substance according to claim 9 in the preparation of a medicament suitable for use in a method according to any one of claims 1-6.

11. A pharmaceutical composition for administration to a mammalian subject, the composition comprising: a therapeutic drug; a $P2X_7$ receptor stimulating substance; and a physiologically acceptable carrier, diluent or excipient.

12. A method of making a pharmaceutical composition comprising the step of combining in admixture a therapeutic drug, a $P2X_7$ receptor stimulating substance, and a physiologically acceptable carrier, diluent or excipient.

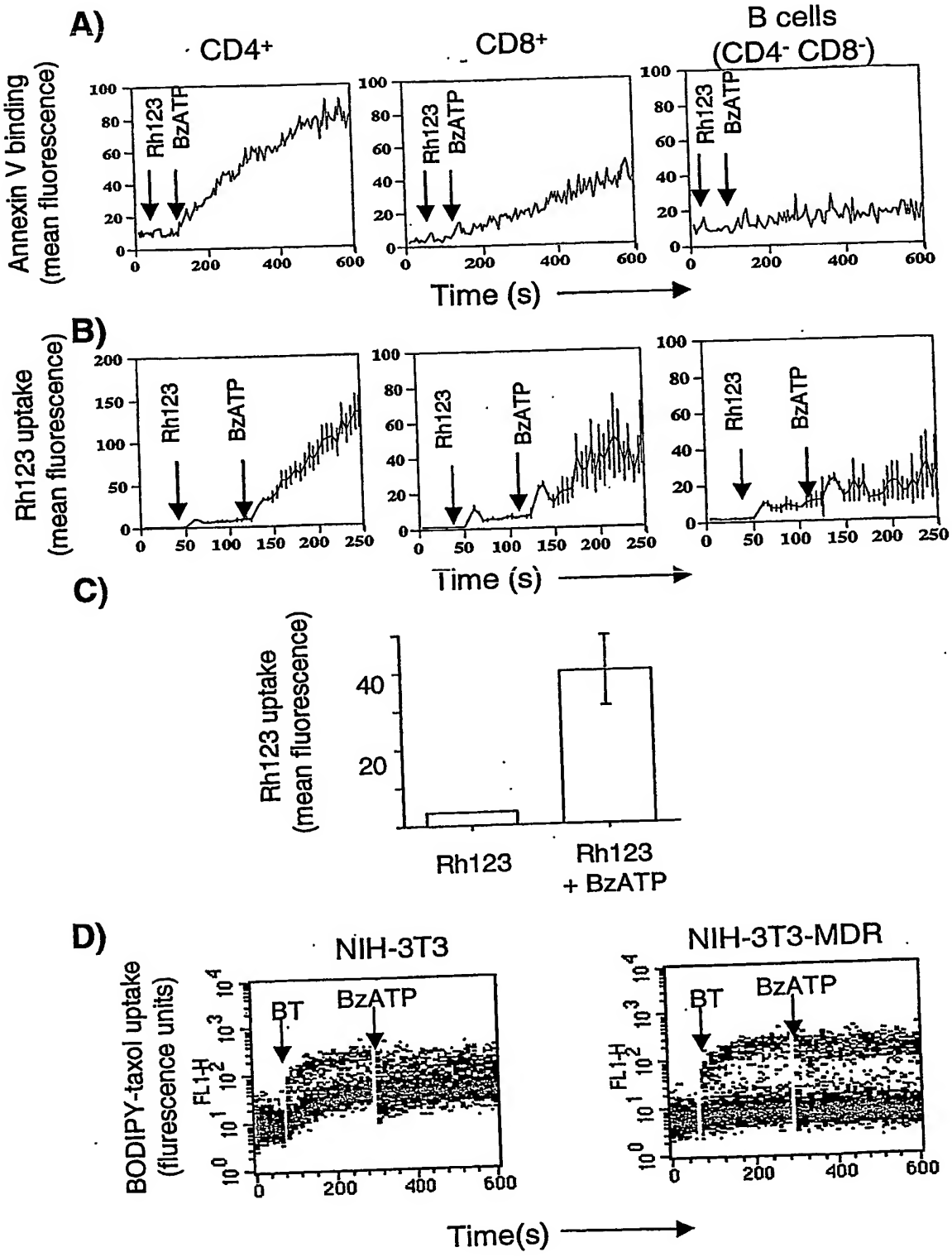
13. A method of inhibiting the action of a cell membrane efflux protein, the method comprising the step of contacting a cell expressing a $P2X_7$ receptor with a substance which causes activation of the $P2X_7$ receptor.

ABSTRACT

Title: Improvements in or Relating to Concentration of Intracellular Agents

Disclosed is a method of increasing the effective intracellular concentration of a therapeutic molecule within a cell expressing a $P2X_7$ receptor, the method comprising the step of contacting the cell with the therapeutic molecule and with a substance which stimulates the $P2X_7$ receptor.

Fig. 1


A)
CD4+
CD8+
B cells
(CD4- CD8-)
Annexin V binding
(mean fluorescence)
Rh123
BzATP
Time (s)
B)
Rh123 uptake
(mean fluorescence)
Rh123
BzATP
Time (s)
C)
Rh123 uptake
(mean fluorescence)
Rh123
Rh123
+ BzATP
D)
NIH-3T3
NIH-3T3-MDR
BODIPY-taxol uptake
(fluorescence units)
FL1-H
BT
BzATP
Time(s)

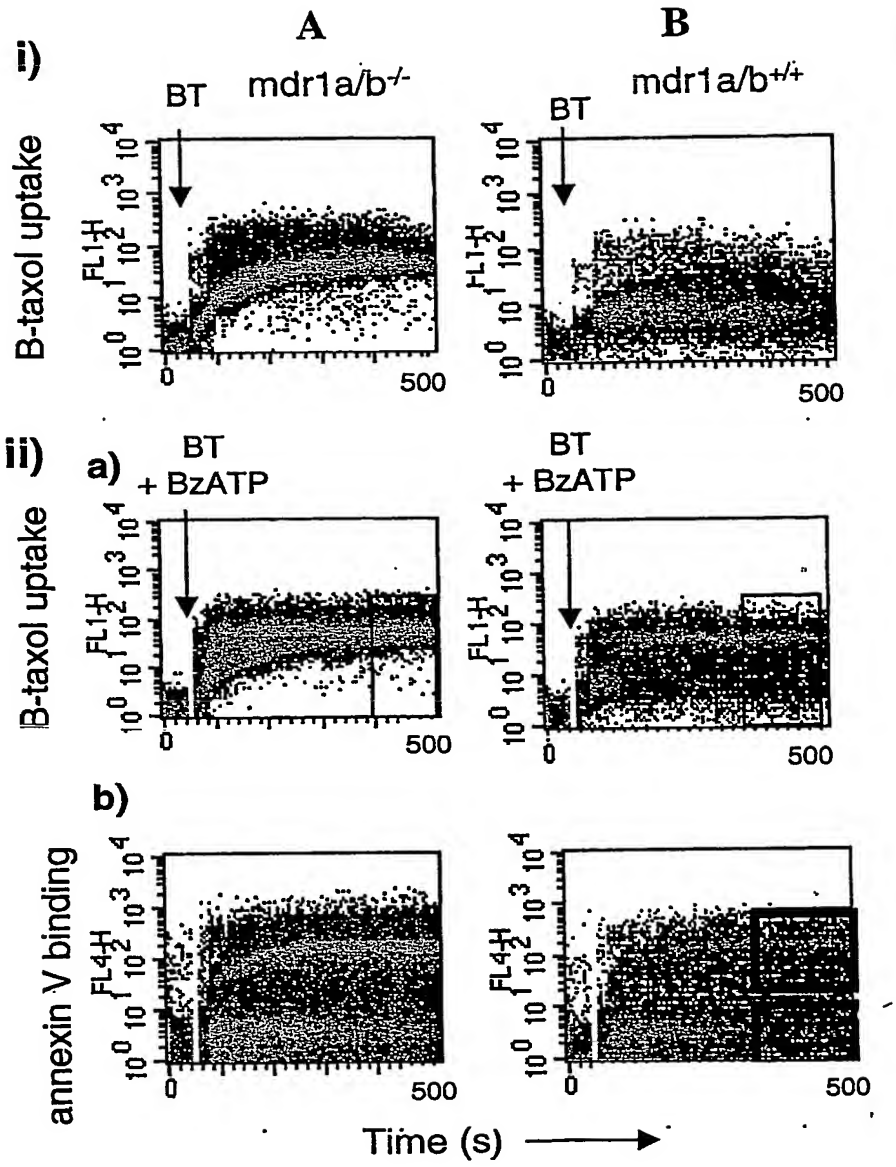

A
B
i)
mdr1a/b-/-
mdr1a/b+/+
BT
B-taxol uptake
FL1-H
ii)
a)
BT
+ BzATP
b)
annexin V binding
FL4-H
0
500
Time (s)

Fig. 2

Fig. 3

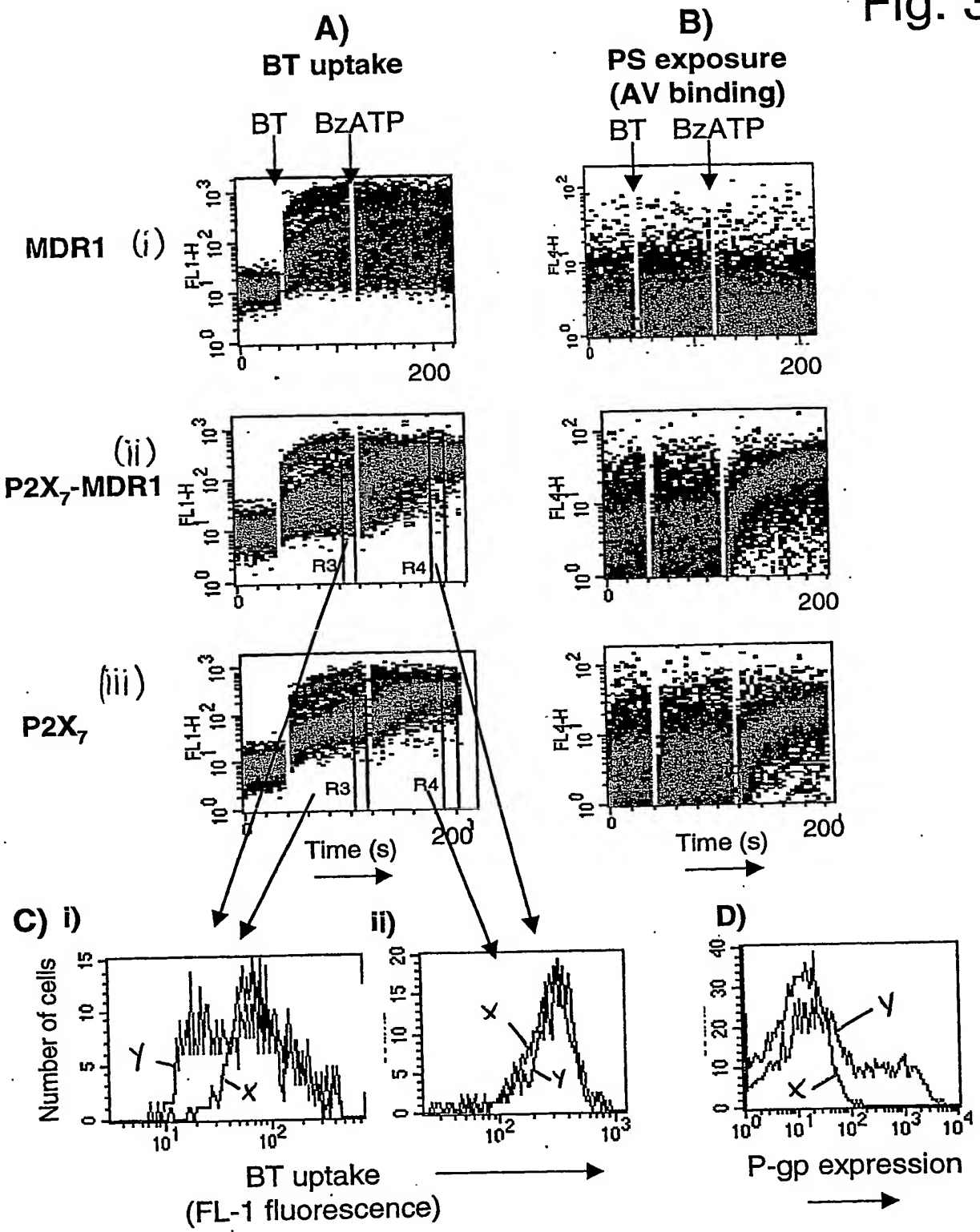

A)
BT uptake
B)
PS exposure
(AV binding)
BT
BzATP
MDR1 (i)
(ii)
P2X7-MDR1
(iii)
P2X7
FL1-H
FL4-H
R3
R4
200
Time (s)
C) i)
ii)
D)
Number of cells
X
Y
BT uptake
(FL-1 fluorescence)
P-gp expression

Fig. 4

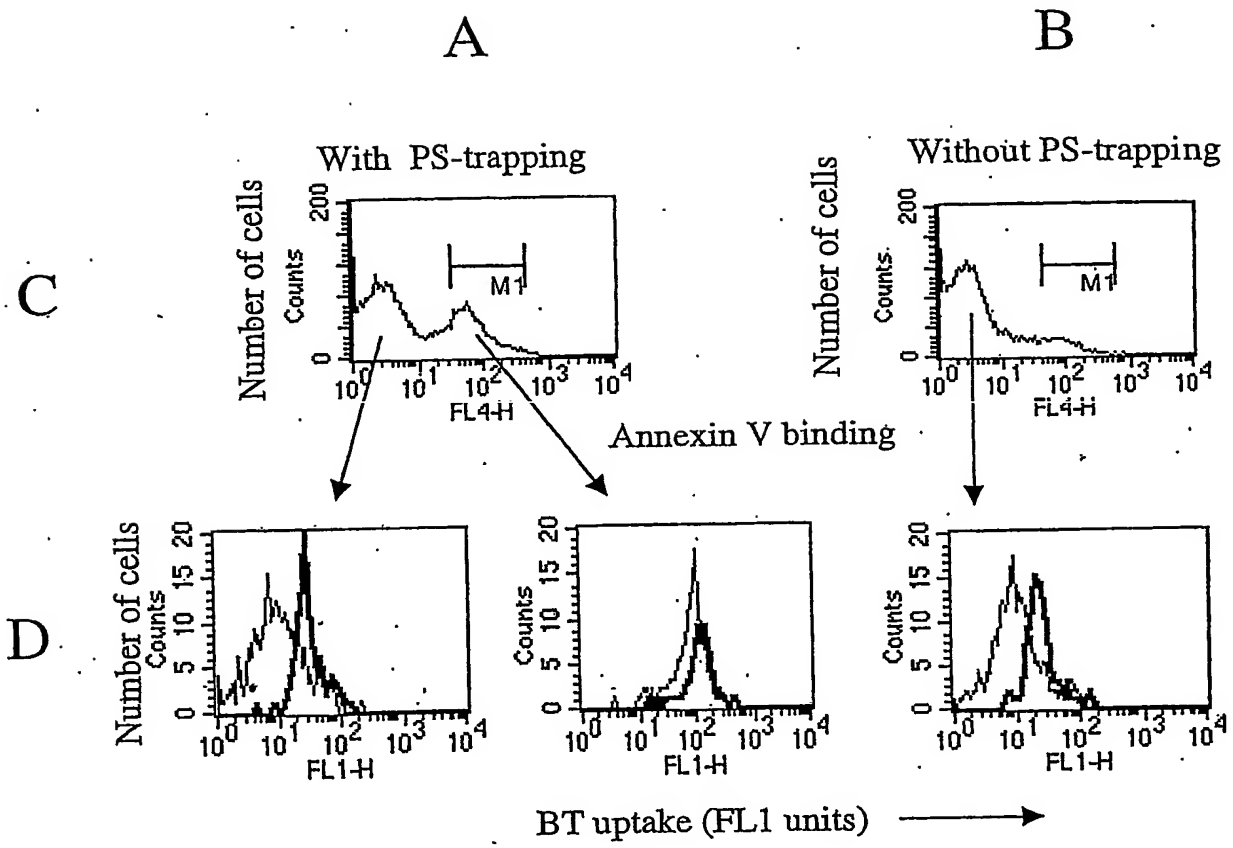
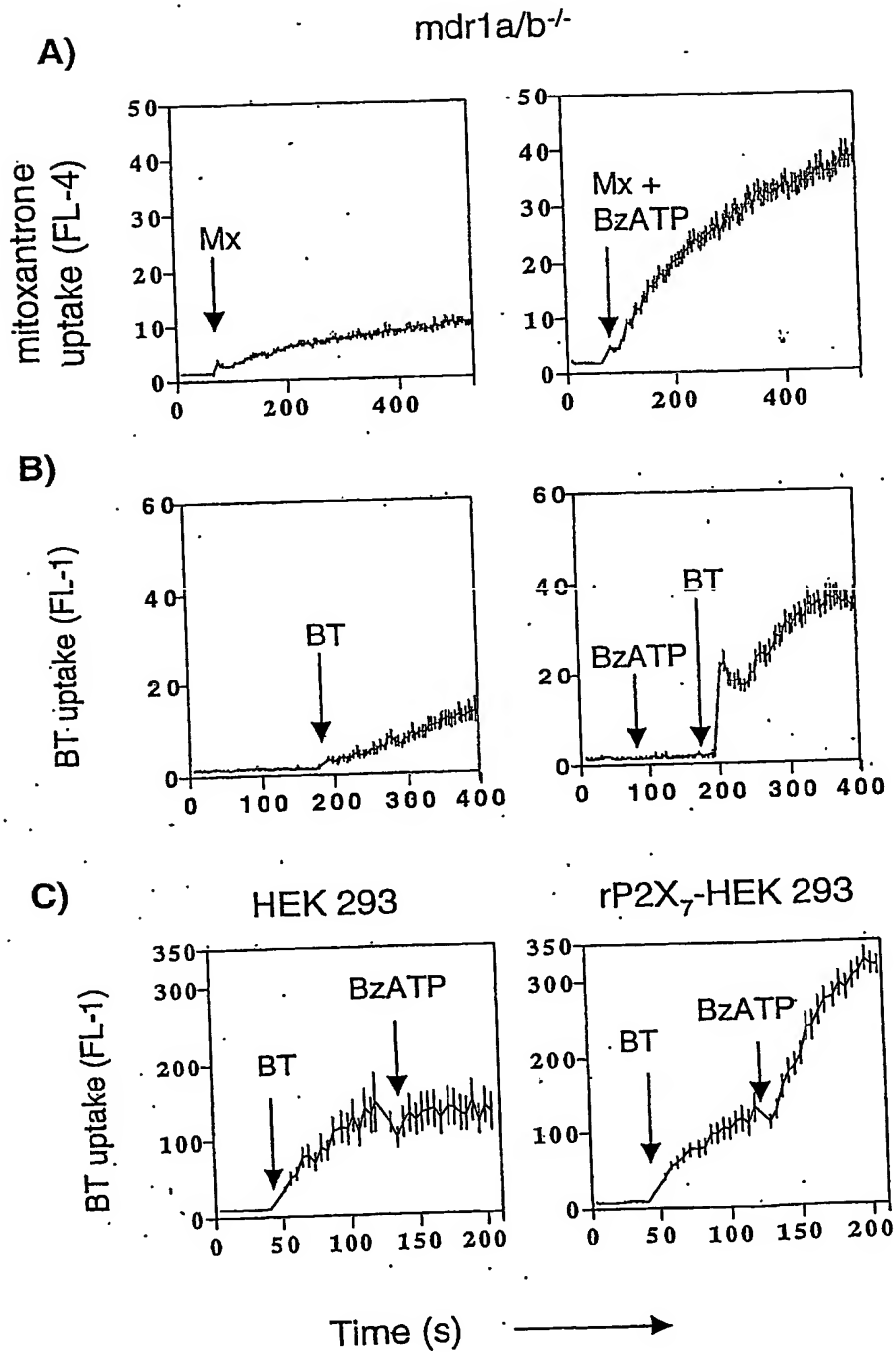

A
B
C
D
With PS-trapping
Without PS-trapping
Number of cells
Counts
M1
FL4-H
Annexin V binding
FL1-H
BT uptake (FL1 units)

Fig. 5


A)
mdr1a/b-/-
mitoxantrone uptake (FL-4)
Mx
Mx + BzATP
B)
BT uptake (FL-1)
BT
BzATP
C)
HEK 293
rP2X7-HEK 293
BT uptake (FL-1)
BT
BzATP
Time (s)